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In vitro regulation of lipogenesis in turkey liver explants

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A mechanical tissue chopper was used to obtain liver explants (35–75 mg) from 2–3-week old turkeys to determine both tissue sensitivity and metabolic effects of adrenergic agonists (isoproterenol, propranolol, norepinephrine and phenoxybenzamine). A previously noted catecholamine induced decrease in *in vitro* lipogenesis in chicken liver explants was also noted in turkey liver explants. Thus, one set of control points for *in vitro* regulation is under control of the cAMP system. Preincubation of slices (1 hr) with propranolol blocked the inhibition of lipogenesis caused by alpha and beta-adrenergic agonists (arterenol or isoproterenol) during a subsequent 1-hr incubation. Preincubation of slices with either of these agonists decreased lipogenesis even following an extensive washout. Inhibition could be overcome with propranolol, a beta adrenergic antagonist.

Key words: Lipogenesis; Turkeys; Metabolic regulation; Isoproterenol; Propranolol; Norepinephrine; Phenoxybenzamine.

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Introduction

Even though several groups studied lipid and carbohydrate metabolism in avian liver over the past 30 years, O'Hea and Leveille (1968) and Goodridge (1968a,b) provided much of the early information on *in vitro* lipogenesis in chicken liver explants. Later work (Tanaka *et al.*, 1979, 1983a,b; Calabotta *et al.*, 1983) expanded the base of knowledge provided by the earlier work. In contrast, I am unaware of any work that describes the regulation of lipogenic pathways in the domestic turkey. This avian species has been bred and selected over the years to have exaggerated breast muscle development. It can be assumed that, because of this muscle development, lipid metabolism may be rudimentary or nonexistent.

Many of the early, and some of the later studies, used techniques described by either Goodridge and Ball (1966) or O'Hea and Leveille (1968). These techniques were, in reality, derived from methods described by DeLuca and Cohen (1964). Briefly, Krebs–Ringer bicarbonate buffer (KRB) was the buffer of choice because its ionic composition approximates mammalian serum. A more recent study showed that both high (Earle's salts) and low bicarbonate based (Hanks' salts) buffers resulted in nearly identical rates of *in vitro* metabolism (Rosebrough and Steele, 1987). Furthermore, both of these salts can be obtained commercially. Thus, large amounts of buffer can be obtained at one time to avoid batch to batch variation. This kind of variation may be inherent in all studies using "home-made" buffer solutions.

Substrates and concentrations used during studies of *in vitro* metabolism have also received

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little attention. Routinely, investigators have used [U-^{14}C]glucose and either [1-^{14}C] or [2-^{14}C]acetate as substrates. Tritiated H_2O has also been successfully used as an indicator of total acetyl group addition during *de novo* lipogenesis. Using [U-^{14}C]glucose may present a problem because the liver is a site of both glycogen degradation and net glucose production (gluconeogenesis). These two processes result in a change in the concentration of intrahepatic and medium glucose pools and therefore, changes in the specific activity of these pools. Acetate then becomes the substrate of choice although the concentration used may be critical if a labile two-carbon pool exists in avian liver; however, the problem of pool dilution can be overcome by using a high concentration of acetate. In addition, the rate of acetyl addition to an existing carbon skeleton has been shown to be sensitive to additions of N^6,O^2-dibutyryl 3′,5′ cyclic adenosine monophosphate (Bt_2cAMP; Allred and Roehrig, 1972). Logically, those hormones associated with cAMP production (peptide and catecholamine) are candidates as regulators of lipogenesis.

The numerous reports concerning *in vitro* measurement of metabolic activities present no systematic protocol for incubation conditions such as time, substrate choice or concentration or points of regulation independent of the physiological state of the donor animal. This presentation describes optimization of methods used for the *in vitro* measurement of lipid metabolism in turkey liver explants and an investigation of points of regulation of hepatic lipid metabolism in the turkey.

Materials and Methods

General

Livers were obtained from 14–28-day old turkeys initially maintained on diets containing 30% protein. Two days before sampling, the turkeys were switched to a diet containing 21% protein. This type of diet supports a high rate of lipogenesis (Rosebrough and Steele, 1985). Turkeys were allowed to consume both feed and water on an *ad libitum* basis. Turkeys were kept in electrically heated battery-brooders (eight turkeys/pen) in an environmentally controlled room (22°C). A 12-hr light (0600–1800 hr), 12-hr dark (1800–0600) cycle was maintained. The turkeys were killed by decapitation at 0900 hr to minimize possible diurnal variation.

Livers were excised and washed with 0.155 M NaCl to remove blood and debris and then placed in chilled phosphate buffered saline (PBS; pH 7.4). The livers were then sliced with a MacIlwain tissue chopper (45–65 mg). Routinely, 24–48 explants could be obtained from a single liver in 5 min. The explants were then placed in 75 mm Petri dishes containing chilled PBS and randomly allocated to *in vitro* treatments such that all treatments were represented by explants from a single liver. All incubations contained 3 ml volumes of Hanks' balanced salts (HBSS) containing 25×10^{-3}M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid; pH 7.4) and were conducted in 25-ml Erlenmeyer flasks at 37°C under a 95% O_2–5% CO_2 atmosphere (obtained by gassing vessels for 30 sec). The incubations were conducted also in the presence of 10^{-2} M [2-^{14}C]sodium acetate that served as the lipogenic substrate in all experiments. After a 2 hr incubation period, the explants from all experiments were placed in 10 ml of 2:1 chloroform:methanol for 18 hr. The extract was partitioned into a chloroform and aqueous phase with 2 ml of 0.88% KCl (Folch *et al.*, 1957). The chloroform phase was evaporated to dryness and lipids were dispersed in scintillation fluid. Radioactivity was measured by liquid scintillation spectroscopy. Treatment replicates were derived from four to six turkeys.

Specific experiments

Experiment 1. *Characterization of regulation of* in vitro *lipogenesis.* This experiment was designed to study possible points of regulation of lipogenesis in turkey liver. We wished to demonstrate that lipogenesis in the turkey can be mediated through 3′-5′ cyclic adenosine monophosphate and as well as through inhibition of ATPase and uncoupling of the reduction of NADPH. The following compounds were selectively added to incubations at 10^{-3} M concentrations; phenazine methosulfate (PMS; an electron acceptor and uncoupling agent of reduced nicotinamide adenine dinucleotide), ouabain (an APTase inhibitor that may also block citrate translocation from the mitochondrion) and N^6,O^2-dibutyryl 3′,5′ cyclic adenosine monophosphate (Bt_2cAMP; a cAMP analog that inactivates acetyl CoA carboxylase or phosphofructokinase).

Experiment 2. *Characterization of adrenergic regulation of* in vitro *lipogenesis.* Experiment 1 confirmed that, in this *in vitro* incubation system, cAMP was a potent inhibitor of lipogenesis. The next task was to affirm the existence of a single, functional class of adrenergic receptor in our system. Initially, quadruplicate explants from each of four to six turkeys were incubated for 2 hr in 10^{-8}–10^{-4} M concentrations of alpha and beta-adrenergic agonists (arterenol and isoproterenol bitartrate respectively). The alpha and beta-adrenergic agonists were incubated in either 0 or 10^{-4} M concentrations of propranolol (a known antagonist to

beta-adrenergic agonists) or phentolamine (a known antagonist to an adrenergic agonist) to determine if two separate classes of adrenergic receptors exist in turkey liver.

Quadruplicate explants from each of four turkeys were then incubated for 2 hr in 10^{-10}–10^{-6} M concentrations of the beta-adrenergic agonist, isoproterenol bitartrate. In addition, explants were incubated in either 0 or 10^{-6} M propranolol. The lower concentration of propranolol was chosen because 10^{-4} M propranolol inhibited lipogenesis and acted like an adrenergic agonist, not like an antagonist as originally proposed. A lower concentration of isoproterenol was chosen because 10^{-8} M isoproterenol decreased lipogenesis to one-half of the control value.

Experiment 3. *Adrenergic agonist interactions.* This experiment was divided into three sections to study various adrenergic hormone interactions *in vitro*. The purpose of the experiments in the first section was to determine the effect of a beta-adrenergic agonist during a preincubation period on the response to the beta-adrenergic agonist or antagonists during subsequent incubation. In the first portion of this experiment, explants were incubated in the presence of 0 or 10^{-6} M isoproterenol or 10^{-6} M propranolol for 1 hr and then either 10^{-6} M isoproterenol or 10^{-6} M propranolol was added along with 10^{-2} M [2-^{14}C]sodium acetate for an additional hour.

In the second portion of this experiment, explants were incubated in HBSS for 1 hr + 0, 10^{-6} M isoproterenol or 10^{-6} M propranolol, washed 3 times with HBSS and then reincubated in 10^{-6} M isoproterenol along with 10^{-2} M [2-^{14}C]sodium acetate for an additional hour. Also, the purpose of the second portion of the experiment was to determine the effect of a beta-adrenergic antagonist during a preincubation period on the response to either an alpha-adrenergic or beta-adrenergic agonist in the following incubation period. Explants were incubated 1 hr + 0 or 10^{-6} M propranolol, washed 3 times with HBSS and then reincubated for 2 hr in 10^{-10}–10^{-6} M concentration of the alpha-adrenergic agonist arterenol or the beta-adrenergic agonist isoproterenol. The last portion of experiment 3 was designed to investigate the kinetics of the agonist–antagonist relationship over different exposure periods.

Statistics

In each experiment, all treatments were randomized across explants obtained from a single liver. Treatment means were derived from turkeys pooled across treatments. Thus, the replicate was the turkey. Animal variation was minimized by transforming all values to their respective natural logs before analyses. Untransformed data are reported, however. Data were analyzed by analyses of variance according to the general linear models (Remington and Schork, 1970).

Results and Discussion

Previous work from this laboratory (Rosebrough and Steele, 1987) indicated that the *in vitro* system described in this presentation was viable for at least 6 hr in the presence of the original incubation medium. Therefore, we feel that the preincubation and incubation periods chosen for the present studies (1 and 2 hr respectively) did not present a time increment in which tissue viability would be suspect.

The results from experiment 1 illustrate three distinct points along the metabolic pathway leading to fat synthesis that can be probed to describe regulation of *in vitro* lipogenesis in avian liver explants (Fig. 1). Ouabain, a noted inhibitor of ATPase, lowered ($P < 0.05$) lipogenesis as did PMS, an uncoupling agent. When combined, both compounds resulted in the greatest degree of inhibition noted in this portion of the study ($P < 0.05$). Although cAMP also depressed lipogenesis ($P < 0.05$), the combination of cAMP and theophylline in previous studies was no more effective than cAMP alone and, likewise, the combination of PMS and either cAMP or ouabain did not decrease lipogenesis any more than PMS alone. Although we demonstrated a depression in fatty acid synthesis in the presence of 10^{-3} M cAMP in previous experiments (Rosebrough and Steele, 1987), it is always necessary to establish that inhibition in the presence of cAMP is

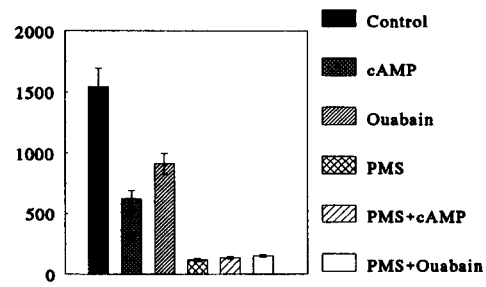

Fig. 1. *In vitro* regulation of lipogenesis in turkey liver explants. Explants were incubated for 2 hr in 25-ml Erlenmeyer flasks in HBSS containing 20 mM HEPES, and 10 mM [2-^{14}C]sodium acetate. Five millimolar concentrations of the following compounds were selectively added to incubations; phenazine methosulfate (PMS; an electron acceptor that may uncouple NADPH production), ouabain (an APTase inhibitor which may also block energy-dependent citrate translocation from the mitochondrion) and N^6,O^2-dibutyryl 3′,5′ cyclic adenosine monophosphate (Bt_2cAMP; a cAMP analog that readily penetrates the lipid bilayer of cells). Values presented are nmoles acetate per 100 mg liver ($n = 4$; experiment 1).

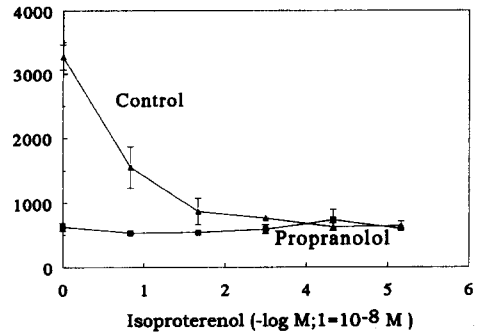

Fig. 2. Effect of a beta adrenergic agonist on *in vitro* lipogenesis. Explants were incubated for 2 hr in 25-ml Erlenmeyer flasks in HBSS containing 20 mM HEPES, and 10 mM [2-^{14}C]sodium acetate and 10^{-4}–10^{-8} M concentrations of the beta adrenergic agonist isoproterenol + 0 or 10^{-4} M propranolol. Values presented are nmoles acetate per 100 mg liver ($n = 4$; experiment 2).

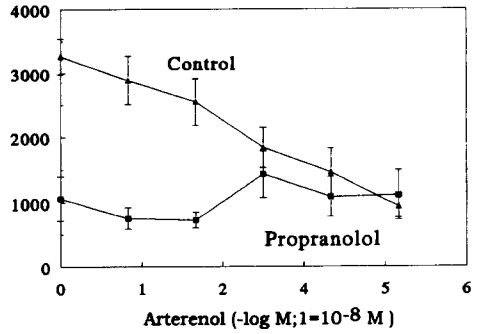

Fig. 4. Effect of an alpha adrenergic agonist on *in vitro* lipogenesis. Explants were incubated for 2 hr in 25-ml Erlenmeyer flasks in HBSS containing 20 mM HEPES and 10 mM [2-^{14}C]sodium acetate and 10^{-4}–10^{-8} M concentrations of the alpha adrenergic agonist arterenol +0 or 10^{-4} M propranolol. Values presented are nmoles acetate per 100 mg liver ($n = 4$; experiment 2).

always greater than that noted for an equal concentration of a specific adrenergic agonist. This finding establishes that regulation occurs between binding of the hormone to a specific receptor and the ultimate generation of cAMP. Furthermore, the description of inhibition of specific metabolic events (ATPase and oxidative coupling) provides evidence that explants have more than one functional metabolic pathway and that fat synthesis noted in the present study is not some artifact of our *in vitro* system. We feel that this rationalization should be applied to all experiments purported as involving the binding of an adrenergic agonist and some metabolic event. The inhibition of acetate utilization for *de novo* lipogenesis noted in the presence of 10^{-3} M Bt_2cAMP coincides with the work of Allred and Roehrig (1972) and corroborates our previous findings of such results in chickens (Rosebrough and Steele, 1987).

Experiment 2 was designed to characterize adrenergic hormone receptors in turkey liver by noting lipogenesis in the presence of either alpha or beta-adrenergic agonists. Figures 2 and 3 illustrate a comparison between the effects of different concentrations of the beta adrenergic agonist isoproterenol and the beta adrenergic antagonist propranolol. Figure 2 shows that 10^{-6} M isoproterenol resulted in maximum inhibition of lipogenesis. Surprisingly, 10^{-4} M propranolol did not block the inhibition of isoproterenol at any concentration. In fact, this concentration of propranolol acted like a beta adrenergic agonist by inhibiting lipogenesis. Figure 3 shows that a lower concentration of propranolol (10^{-6} M) would indeed inhibit lipogenesis when lower concentrations of isoproterenol were used (10^{-10}–10^{-6} M). These two sets of observations provide an interesting contrast to results from chicken experiments (Rosebrough and Steele, 1987). Comparisons of data sets from chicken and turkey experiments show that the turkey hepatocyte is more responsive than the chicken hepatocyte to

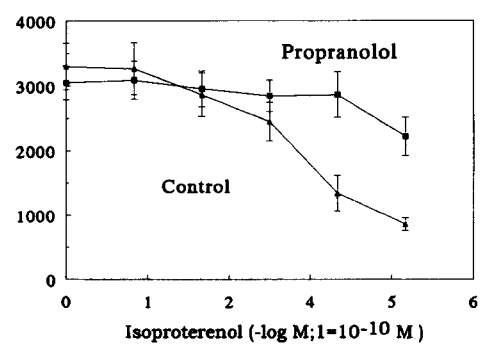

Fig. 3. Effect of a beta adrenergic agonist on *in vitro* lipogenesis. Explants were incubated for 2 hr in 25-ml Erlenmeyer flasks in HBSS containing 20 mM HEPES, and 10 mM [2-^{14}C]sodium acetate and 10^{-6} to 10^{-10} M concentrations of the beta adrenergic agonist isoproterenol +0 or 10^{-6} M propranolol. Values presented are nmoles acetate per 100 mg liver ($n = 4$; experiment 2).

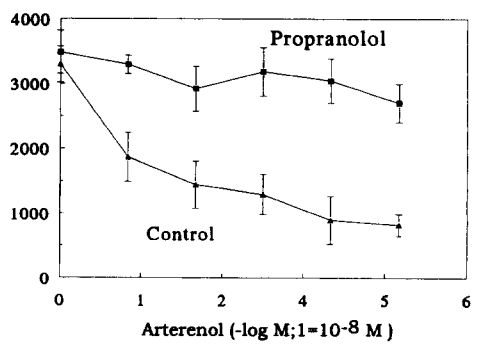

Fig. 5. Effect of an alpha adrenergic agonist on *in vitro* lipogenesis. Explants were incubated for 2 hr in 25-ml Erlenmeyer flasks in HBSS containing 20 mM HEPES and 10 mM [2-^{14}C]sodium acetate and 10^{-4}–10^{-8} M concentrations of the alpha adrenergic agonist arterenol +0 or 10^{-6} M propranolol. Values presented are nmoles acetate per 100 mg liver ($n = 4$; experiment 2).

catecholamines. Furthermore, 10^{-4} M propranolol inhibits the effect of isoproterenol on the chicken hepatocyte, but acts like isoproterenol on the turkey hepatocyte.

Figures 4 and 5 illustrate the effect of the alpha adrenergic agonist, arterenol, on lipogenesis. Although the initial increment of arterenol (10^{-8} M) resulted in a sharp inhibition of lipogenesis, each additional increment also resulted in a further decrease in lipogenesis. Again, 10^{-4} M propranolol did not overcome the effect of arterenol, but acted like an adrenergic agonist instead. Lowering the concentration of propranolol to 10^{-6} M restored the effect of propranolol as an adrenergic antagonist, however. At this concentration, propranolol inhibited the effect of arterenol on lipogenesis, resulting in lipogenic rates similar to control values at all concentrations of arterenol. Although the slopes for both agonists were different ($P < 0.05$), this effect was due to the effects of the hormones at low concentrations and not to differences in maximum inhibition (V_{max}) noted in the presence of either 10^{-5} M isoproterenol or arterenol. The latter observation provides partial evidence for the lack of specific alpha- and beta-adrenergic receptors in avian liver. Propranolol, a purported beta-adrenergic antagonist, blocked the inhibition of lipogenesis noted in the presence of 10^{-8}–10^{-4} M concentrations of an alpha adrenergic agonist.

Figure 6 illustrates the questionable effect of phentolamine, an alpha-adrenergic antagonist, on the inhibition of lipogenesis noted in the presence of the alpha-adrenergic agonist arterenol. Although certain points on the dose–response curves were different, the overall slopes in the presence and absence of phentolamine were similar. Together, Figs 2–6 can be

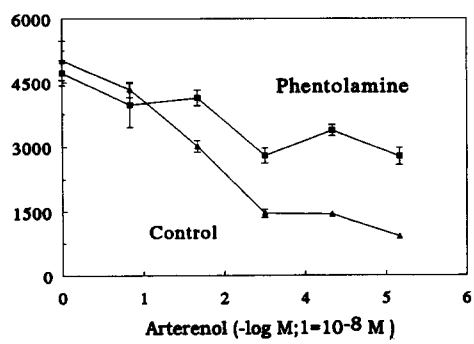

Fig. 6. Effect of an alpha adrenergic agonist (arterenol) +0 or 10^{-4} M phentolamine (an alpha-adrenergic antagonist) on *in vitro* lipogenesis in turkey liver explants. Explants were incubated in the presence of 10^{-2} M [2-^{14}C]sodium acetate and 10^{-8}–10^{-4} M concentrations of the agonist. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 2).

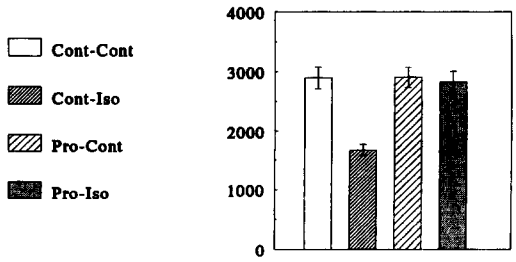

Fig. 7. Effect of preincubation on inhibition of *in vitro* lipogenesis in turkey liver explants. Explants were preincubated +0 or 10^{-6} M propranolol (Pro) and 10^{-2} M [2-^{14}C]sodium acetate for 1 hr and then for an additional hr in the presence of and 0 or 10^{-6} M isoproterenol (Iso). Control (no agonist or antagonist) is labeled as Cont. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 3).

taken as clear evidence of a single class of adrenergic agonist in the livers of turkeys used in the present study.

Preincubation of explants in the presence of 0 or 10^{-6} M isoproterenol changed tissue sensitivity to isoproterenol during a subsequent 1-hr incubation (Fig. 7). It is important to remember that, in this experiment, the explant was incubated in the presence of propranolol for 2 hr and isoproterenol for 1 hr. Under these conditions, it was noted that propranolol could block the effect of isoproterenol on lipogenesis. The data also indicate no effect of propranolol on lipogenesis. Figure 8 summarizes an incubation scheme opposite to Fig. 7. Preincubation in the presence of isoproterenol and then incubation in propranolol resulted in a lipogenic rate less than incubation in the presence of isoproterenol alone. In this respect, propranolol may block further inhibition of lipogenesis, but has no obvious effect upon that portion of the reaction conducted without propranolol.

Experiment 3 was conducted to determine carry over effects of either an adrenergic agonist

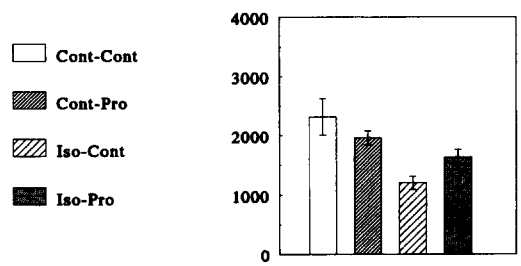

Fig. 8. Effect of preincubation on inhibition of *in vitro* lipogenesis in turkey liver explants. Explants were preincubated +0 or 10^{-6} M isoproterenol and 10^{-2} M [2-^{14}C]sodium acetate for 1 hr and then for an additional hr in the presence of and 0 or 10^{-6} M propranolol. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 3).

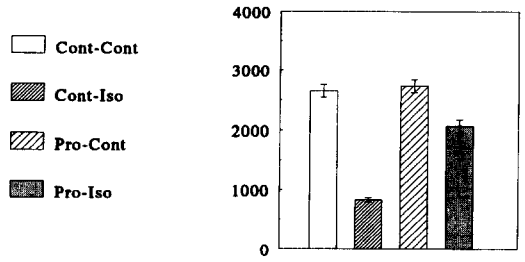

Fig. 9. Effect of preincubation on inhibition of *in vitro* lipogenesis in turkey liver explants. Explants were preincubated +0 or 10^{-6} M propranolol for 1 hr and then incubated for 1 hr in the presence of 10^{-2} M [2-^{14}C]sodium acetate and 10^{-6} M isoproterenol. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 3).

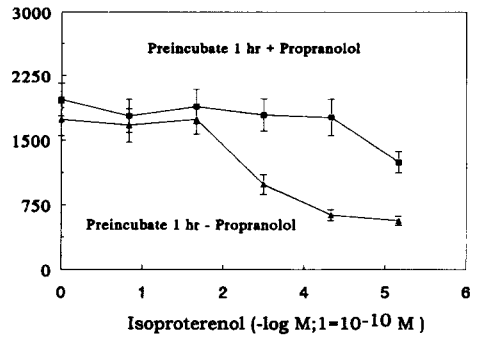

Fig. 11. Effect of preincubation on inhibition of *in vitro* lipogenesis in turkey liver explants. Explants were preincubated +0 or 10^{-6} M propranolol for 1 hr and then incubated for 1 hr in the presence of 10^{-2} M [2-^{14}C]sodium acetate and 10^{-6}–10^{-10} M isoproterenol. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 3).

or antagonist. The above experiment (experiment 2) could be faulted in that agonists and antagonists were present together during some phase of each incubation. Therefore it would be difficult to prove that an agonist/antagonist–receptor complex was stable. Experiment 3 involved first, an incubation in the presence of either the agonist or antagonist and a second incubation in fresh media in the presence either an agonist or antagonist. The second incubation was necessary to remove unbound agonists or antagonists remaining from the first incubation. It could then be stated that effects noticed in the second incubation were due to compounds originally bound during the first incubation.

Preincubation in the presence of propranolol blocked the inhibition of lipogenesis normally caused by isoproterenol (Fig. 9). In contrast, preincubation in the presence of propranolol and incubation without isoproterenol resulted in a lipogenic value similar to controls. It was found that lipogenesis in tissue preincubated for 1 hr in the presence of isoproterenol and then incubated in fresh medium without isoproterenol was still less ($P < 0.01$; Fig. 10) than in control tissue. In fact, this value was almost identical to that for tissue preincubated without isoproterenol but, incubated for 1 hr in the presence of isoproterenol (Fig. 9).

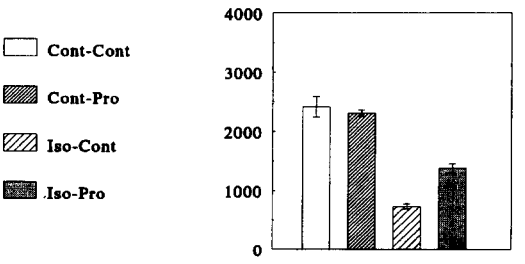

Fig. 10. Effect of preincubation on inhibition of *in vitro* lipogenesis in turkey liver explants. Explants were preincubated +0 or 10^{-6} M isoproterenol for 1 hr and then incubated for 2 hr in the presence of 10^{-2} M [2-^{14}C]sodium acetate and 10^{-6} M propranolol. *In vitro* lipogenesis is expressed as nmoles of [2-^{14}C]sodium acetate incorporated into hepatic fatty acids per 100 mg of tissue ($n = 4$; experiment 3).

The above observations demonstrated the adrenergic hormone–receptor complex was resistant to washout and was stable during a subsequent incubation without any further adrenergic agonist to replenish the complex. Preincubation of tissue in the presence of 10^{-6} M propranolol followed by extensive washing showed that the adrenergic antagonist–receptor complex was as stable as the adrenergic agonist–receptor complex. This preincubation–incubation regimen still blocked the effect of isoproterenol (10^{-10}–10^{-6} M), a beta-adrenergic agonist (Fig. 11) over a wide range of concentrations.

The studies in this report further demonstrate that catecholamines are viable inhibitors of *in vitro* lipogenesis in turkey liver explants. These effects have been described in hepatocytes from other species (Capuzzi *et al.*, 1975; Cramb *et al.*, 1982; Campbell and Scanes, 1985). These results show that hepatocytes are also useful tools to study sensitivity to catecholamines. Certain differences in methodologies (as well as intact tissue versus isolated cells) make comparisons among studies difficult. For example, there is a possibility that either spare beta-adrenergic receptors are unmasked or that further binding ability is gained during hepatocyte isolation techniques (Nakamura *et al.*, 1983).

The present study demonstrates that turkey liver may be more sensitive than chicken liver to catecholamine regulation of lipogenesis. The present study also indicates that even though the domestic turkey has been extensively selected for lean tissue synthesis, the ability to synthesize lipid *de novo* remains intact.

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